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Journal of Cystic Fibrosis 13 (2014) 56 – 62

Journal of Cystic Fibrosis

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Original Article

Investigating the role of matrix components in protection of *Burkholderia cepacia* complex biofilms against tobramycin

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Received 17 May 2013; received in revised form 4 July 2013; accepted 20 July 2013
Available online 8 August 2013

Abstract

Background: *Burkholderia cepacia* complex (Bcc) organisms produce a wide variety of potential virulence factors, including exopolysaccharides (EPS), and exhibit intrinsic resistance towards many antibiotics. In the present study we investigated the contribution of Bcc biofilm matrix components, including extracellular DNA, cepacian and poly-β-1,6-N-acetylglucosamine, to tobramycin susceptibility.
Methods: The *in vitro* bactericidal activity of tobramycin in combination with recombinant human DNase (rhDNase), NaClO and dispersin B was tested against Bcc biofilms.
Results: EPS degradation by NaClO pretreatment and specific PNAG degradation by dispersin B significantly increased the bactericidal effect of tobramycin towards some of the Bcc biofilms tested, including the strains of *Burkholderia cenocepacia*, *B. cepacia* and *Burkholderia metallica*. The presence of rhDNase during biofilm treatment and/or development had no influence on tobramycin activity.
Conclusion: These results suggest that EPS play a role in tobramycin susceptibility of Bcc biofilms and that matrix degrading combination therapy could improve treatment of Bcc biofilm infections.
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Keywords: *Burkholderia cepacia* complex; Biofilm; Tobramycin; Matrix; Cepacian; PNAG

1. Introduction

Chronic *Pseudomonas aeruginosa* lung infections are the primary cause of morbidity and mortality in CF patients [1]. Although *P. aeruginosa* is the dominant pathogen isolated from the chronically infected CF lung, infections with *Burkholderia cepacia* complex (Bcc) species often lead to a more rapid decline in lung function, resulting in increased mortality [2]. Chronic Bcc infections are very difficult to eradicate because of the capacity of Bcc species to cause invasive disease and their high level of intrinsic antibiotic resistance. The production of different exopolymeric substances by mucoid Bcc bacteria provides additional protection against both antibiotics and host immune components [3]. Exopolysaccharides (EPS) produced by *Burkholderia cenocepacia* have been shown to inhibit neutrophil chemotaxis and to reduce the levels of neutrophil derived reactive oxygen species (ROS) [4]. This reduction in ROS levels is caused by the ROS-scavenging capacities of these EPS molecules [4]. Cuzzi et al. observed a protective effect of cepacian, a Bcc specific EPS, against hypochlorite (ClO^-), a highly bactericidal ROS produced by neutrophils. This protective effect was also provided by scavenging, resulting in extensive deacetylation of cepacian followed by the degradation of the polysaccharide backbone [5]. Although cepacian is the most common EPS produced by Bcc species, the production of at least six other polysaccharides, either synthesized alone or in combination with cepacian, has been demonstrated [6]. *Burkholderia vietnamiensis* LMG 10929, used in this study, has been shown not to produce cepacian as main EPS but a polysaccharide containing a fucose residue in its backbone. In addition, the production of poly-β-1,6-N-acetylglucosamine (PNAG), also known as "polysaccharide intercellular adhesin" (PIA), an important virulence factor and major constituent of staphylococcal biofilms, has been

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http://dx.doi.org/10.1016/j.jcf.2013.07.004

documented in several Bcc biofilms [7]. Although it appears to be a minor component compared to cepacian it seems essential for initiating biofilm formation as well as maintaining biofilm integrity. Apart from EPS, another important constituent of the biofilm matrix is extracellular DNA (eDNA). Most research about the contribution of eDNA to biofilm formation and resistance has focused on *P. aeruginosa* in which eDNA appears to be essential for the formation and stabilization of a complex biofilm structure [8]. Witchurch et al. first demonstrated a reduction in *P. aeruginosa* biofilm formation in the presence of DNase. Subsequently, the contribution of eDNA to biofilm formation of a variety of bacterial species, including *Staphylococcus epidermidis* and *Staphylococcus aureus*, was investigated [9–11]. In *P. aeruginosa* and *Salmonella enterica* biofilms the contribution of eDNA to aminoglycoside tolerance has already been shown. By binding divalent cations, a cation-limited environment is created which in turn induces the expression of *pmr* genes conferring resistance towards aminoglycosides [12,13]. In addition, Chiang et al. observed a shielding effect of eDNA in *P. aeruginosa* biofilms, mediating a delayed penetration of aminoglycosides [14]. Still, little is known about the role of eDNA in *Burkholderia* biofilms, although it seems to be essential for cellular aggregation in *Burkholderia thailandensis* [15].

The aim of the present study was to investigate whether Bcc biofilm matrix components, including eDNA, cepacian and PNAG play a role in the protection of biofilm cells against tobramycin. To this end, the bactericidal effect of tobramycin in combination with matrix degrading components (rhDNase, NaClO and dispersin B) was tested against several *in vitro* grown Bcc biofilms and compared with the bactericidal effect of tobramycin alone.

2. Materials and methods

2.1. Bcc strains

Strains used (Table 1) were obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium) or were kindly provided by Dr. P. Vandamme (Ghent University, Belgium). The bacteria were stored in Microbank tubes (Prolab Diagnostics, Richmond Hill, ON, Canada) at −80 °C and transferred twice on Mueller Hinton (MH) (Lab M, Heywood, UK) agar plates before use in any experiment. All cultivations were performed under aerobic conditions at 37 °C.

Table 1
Bcc strains used in the present study.

Strain	Isolation source	Tobramycin MIC (μg/mL)
Burkholderia cenocepacia LMG 16656	CF patient	256
Burkholderia multivorans LMG 13010	CF patient	64
Burkholderia cepacia LMG 1222	Onion	32
Burkholderia anthina LMG 20980	Soil	16
Burkholderia vietnamiensis LMG 10929	Soil	16
Burkholderia metallica LMG 24068	CF patient	64

2.2. Reagents

A highly purified solution of rhDNase (1.0 mg/mL) (Pulmozyme, Genentech Inc., San Francisco, CA, USA) which selectively degrades DNA, was obtained from the pharmacy of the Ghent University hospital (Ghent, Belgium). It was stored between 2 and 8 °C, protected from light. A NaClO solution (4–4.99% available chlorine), was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored between 2 and 8 °C protected from light. Lyophilized dispersin B was obtained from Kane Biotech Inc. (Winnipeg, MB, Canada) and was dissolved in equal volumes of glycerol and 100 mM phosphate buffer pH 5.9, supplemented with 200 mM NaCl. The dispersin B solution was stored at −20 °C.

2.3. Biofilm formation

An inoculum was prepared by suspending bacteria from a pure culture on MH agar in MH broth. The inoculum was standardized at ~10^8 CFU/mL and the wells of a round-bottomed 96-well microtiter plate (TPP, Trasadingen, Switzerland) were filled with 100 μL of this standardized bacterial suspension. Plates were incubated at 37 °C for 4 h without shaking. After 4 h, the supernatant, containing planktonic cells, was aspirated from the wells. Adhered cells were carefully washed with 100 μL of physiological saline (0.9% NaCl) (PS) and 100 μL of fresh sterile MH broth was added. Plates were incubated for another 20 h at 37 °C without shaking.

2.4. Quantification of eDNA in the biofilm matrix

Eppendorf protein LoBind microcentrifuge tubes (1.5 mL) (Eppendorf AG, Hamburg, Germany) were inoculated with 600 μL of a standardized bacterial solution (~10^8 CFU/mL) in cation-supplemented MH broth (0.015% (w/v) $CaCl_2$; 2.0 mM $MgCl_2$) (csMH broth), with or without the addition of rhDNase (50 μg/mL). Experiments were performed in csMH broth as Ca^{2+} and Mg^{2+} are essential for DNase I activity [16]. Tubes were incubated aerobically for 4 h at 37 °C without shaking. After 4 h, the supernatant was gently poured from the tubes to remove non-adhered planktonic cells. Tubes were rinsed with PS followed by the addition of 600 μL of fresh sterile csMH broth, with or without 50 μg/mL rhDNase. After 20 h of additional incubation, a biofilm had formed on the walls of the tubes (Supplementary data, Fig. SI) and these were gently rinsed with PS without disturbing the adherent film. Subsequently, 300 μL of DNA rehydration solution (Promega, Madison, WI, USA) was added and biofilms were dispersed in this solvent by vortexing the tubes.

Biofilm cells were separated from the matrix by centrifugation at 5000 *g* for 10 min at 4 °C. The supernatant was aspirated and filtered through a 0.2 μm cellulose acetate filter (Whatman GmbH, Dassel, Germany) and the amount of eDNA was quantified using the Quantifluor dsDNA System kit (Promega, Madison, WI, USA). eDNA concentrations were normalized to the number of biofilm cells, determined by plate counting after 24 h of biofilm growth.

2.5. Determination of tobramycin MICs

MICs were determined in duplo according to the EUCAST broth microdilution protocol using unsupplemented MH broth. In brief, bacterial suspensions were standardized at ~5 × 10^5 CFU/mL before inoculating a flat-bottomed 96-well plate filled with 100 μL of tobramycin serial dilutions in MH. The range of tobramycin concentrations used was from 2 to 1024 mg/L. Plates were incubated at 37 °C for 20 h and the optical density was determined at 590 nm using a multilabel microtiter plate reader (Envision, Perkin Elmer LAS, Waltham, MA, USA). The MIC is the lowest concentration of antibiotic for which a similar optical density was observed in the inoculated and blank wells.

2.6. Effect of rhDNase on tobramycin susceptibility of 24 h-old Bcc biofilms

Biofilm formation was carried out in csMH broth with or without 50 μg/mL of rhDNase. After 24 h, the supernatant was discarded and biofilms were washed with PS. Subsequently, 100 μL of tobramycin in cation adjusted PS (0.9% NaCl; 0.015% $CaCl_2$) with or without 50 μg/mL of rhDNase was added. A tobramycin concentration of 4XMIC was used as previous research from our group indicated that these concentrations resulted in a substantial bactericidal effect against Bcc biofilms [17]. After 24 h of additional incubation at 37 °C, cell numbers were determined by plate counting.

2.7. Effect of NaClO on tobramycin susceptibility of 24 h-old Bcc biofilms

Biofilm formation was carried out as described above. After 24 h, the supernatant was aspirated from the wells and biofilms were washed with PS. Before treatment with tobramycin (4XMIC in PS), biofilms were exposed to a 15 min pretreatment with NaOCl (60 μM) (pH 7–8) in phosphate buffered saline (PBS; pH 7) or with non-supplemented PBS. The NaClO concentration used was based on the data published by Cuzzi et al. [5]. Subsequently, biofilms were incubated for another 24 h, followed by the determination of cell numbers by plate counting.

2.8. Effect of dispersin B on tobramycin susceptibility of 24 h-old Bcc biofilms

After 24 h of biofilm formation, biofilms were washed with PS and treated either with phosphate buffer (100 mM, pH 5.9), with phosphate buffer supplemented with tobramycin (4XMIC) (pH 7) or with phosphate buffer supplemented with tobramycin (4XMIC) and dispersin B (final concentration of 100 μg/mL) (pH 7). Plates were incubated for an additional 24 h, followed by determination of cell numbers by plate counting.

2.9. Statistical analyses

Microtiter plate biofilm assays were performed in 12 wells per condition and their content was pooled before plate counting. All experiments were repeated three times on three separate occasions (n = 3). Data are represented as means ± SD of the mean. The significance of differences between treatment groups was determined using the Mann–Whitney non-parametric test, with $p < 0.05$ considered as significant.

3. Results

3.1. Quantification of eDNA in biofilm matrix

Biofilms were grown on the walls of Eppendorf tubes for 24 h in the presence or absence of rhDNase (50 μg/mL) (Fig. SI). Treatment with rhDNase alone did not lead to dispersion of the biofilm, as no difference in cell numbers was observed when biofilms were grown in the presence or absence of rhDNase. The eDNA content of the biofilm differs considerably between the six 6 Bcc strains investigated, with *Burkholderia multivorans* LMG 13010 showing the highest amounts (Fig. 1). By adding rhDNase to the growth medium, a significant reduction in the amount of eDNA was observed for *B. multivorans* LMG 13010, *Burkholderia anthina* LMG 20980, *B. cepacia* LMG 1222 and *B. cenocepacia* LMG 16656 (Fig. 1). No such difference was observed after rhDNase treatment for biofilms of *Burkholderia metallica* LMG 24068 and *B. vietnamiensis* LMG 10929.

3.2. Effect of rhDNase on tobramycin susceptibility of 24 h-old Bcc biofilms

rhDNase (50 μg/mL) alone had no effect on the viability or dispersion of Bcc biofilms (Supplementary data, Fig. SII). The bactericidal activity of tobramycin was tested in three experimental conditions. Firstly, tobramycin (4XMIC) was added to a biofilm formed in the presence of 50 μg/mL rhDNase. Secondly, tobramycin (4XMIC) in combination with 50 μg/mL rhDNase was added to a biofilm formed in the presence of 50 μg/mL rhDNase. Finally, tobramycin (4XMIC) was added to a biofilm formed in the absence of rhDNase. No additional bactericidal effect, caused by the presence of rhDNase during biofilm treatment and/or during biofilm formation, was observed (Table 2).

3.3. Effect of NaClO pretreatment on tobramycin susceptibility of 24 h-old Bcc biofilms

A short pretreatment of the biofilms with a 60 μM NaClO solution (pH 7–8) had no bactericidal effect on its own for all Bcc biofilms tested (Fig. SII). For some Bcc species, tobramycin (4XMIC) added to NaClO pretreated biofilms resulted in a significantly increased bactericidal effect compared to tobramycin added to PBS pretreated biofilms (Table 3). The most pronounced effect of NaClO pretreatment was noticed for *B. metallica* LMG 24068, for which an additional 1.42 log reduction in cell numbers was observed compared to tobramycin treatment alone. No increased bactericidal effect of tobramycin was observed in NaClO pretreated *B. anthina* LMG 20980 and *B. vietnamiensis* LMG 10929 biofilms.

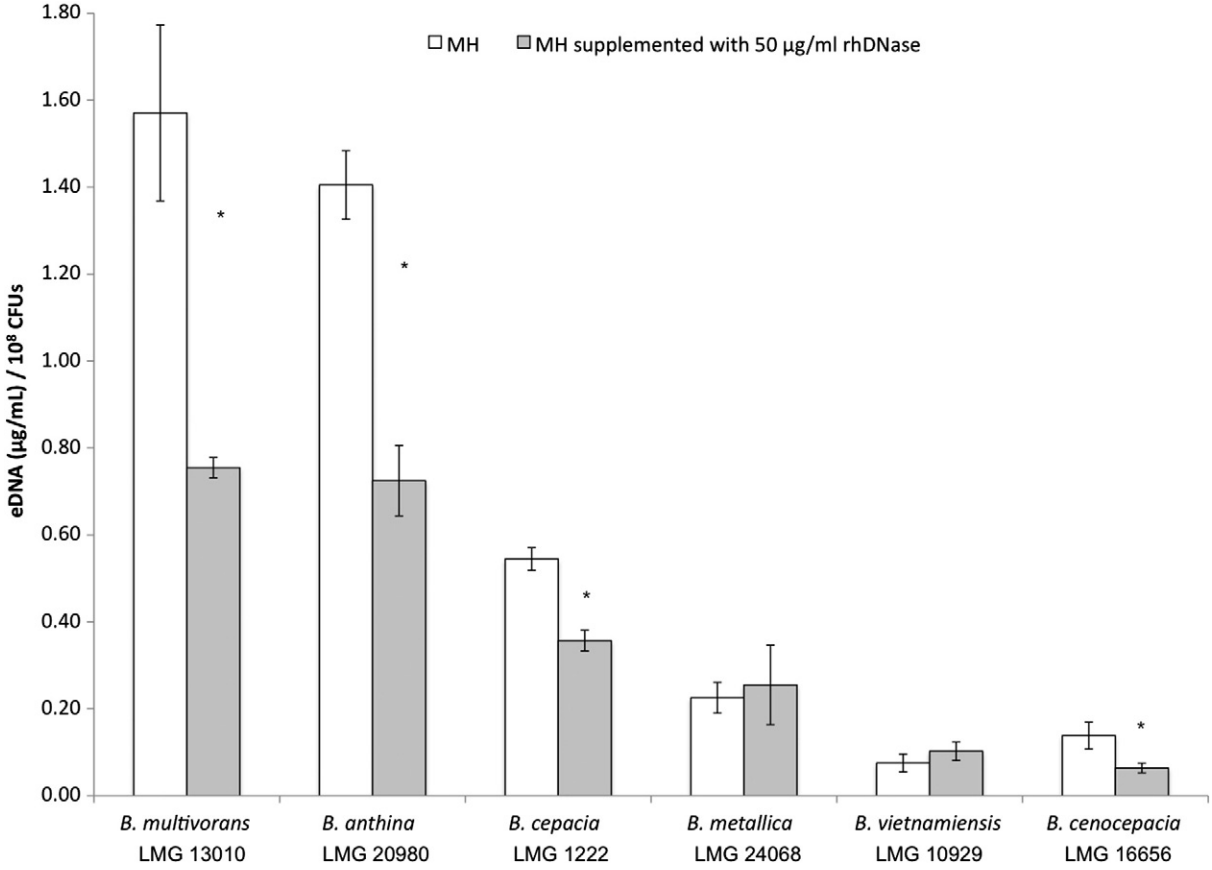

Fig. 1. Determination of cell numbers and quantification of eDNA in *Burkholderia* biofilms. Average amount of eDNA present in the biofilm matrix after 24 h of growth in the presence and absence of rhDNase. The amount of eDNA was normalized for the number of cells adhered to the tube walls. Error bars represent standard deviations of 3 independent measurements (n = 3). *: significantly different compared to biofilms grown in the absence of rhDNase ($p < 0.05$).

3.4. Effect of dispersin B on tobramycin susceptibility of 24 h-old Bcc biofilms

Dispersin B at concentrations of 100 µg/mL had no bactericidal effect on any Bcc biofilm tested (Fig. SII). The bactericidal effect of tobramycin dissolved in phosphate buffer (100 mM, pH 5.9) (Table 4; first row) was significantly reduced compared to the bactericidal effect of tobramycin dissolved in PS (Tables 2 and 3; first row). Still, for three Bcc isolates, *B. cenocepacia* LMG 16656, *B. cepacia* LMG 1222 and *B. metallica* LMG 24068, dispersin B significantly increased the bactericidal effect of tobramycin.

4. Discussion

In the present study we investigated the role of different Bcc biofilm matrix components in the protection against the antimicrobial activity of tobramycin. Tobramycin, a positively charged aminoglycoside antibiotic, is likely to bind to EPS or eDNA, which will decrease the bioavailability of free tobramycin.

Pulmozyme (a highly purified solution of rhDNase) was approved in 1993 by the US Food and Drug Administration (FDA) for the treatment of CF lung disease. rhDNase degrades eDNA, which is present in large amounts in the sputum of CF patients, resulting in reduced sputum viscosity and improved

Table 2
Average log reductions (±SD) after tobramycin (4XMIC) treatment.

Biofilm formation	Biofilm treatment	*B. cenocepacia* LMG 16656	*B. multivorans* LMG 13010	*B. cepacia* LMG 1222	*B. anthina* LMG 20980	*B. vietnamiensis* LMG 10929	*B. metallica* LMG 24068
csMH broth	Tobramycin	3.87 (±0.13)	3.01 (±0.13)	3.14 (±0.37)	4.00 (±0.33)	4.46 (±0.10)	3.55 (±0.18)
csMH broth + rhDNase	Tobramycin	3.75 (±0.19)	3.05 (±0.30)	2.89 (±0.39)	3.70 (±0.33)	4.50 (±0.11)	3.60 (±0.16)
csMH broth + rhDNase	Tobramycin + rhDNase	3.66 (±0.18)	2.78 (±0.08)	2.74 (±0.37)	3.44 (±0.24)	4.17 (±0.16)	3.52 (±0.18)

Tobramycin was added to biofilms formed in csMH broth (control), biofilms formed in csMH broth in the presence of rhDNase and biofilms formed and treated in the presence of rhDNase. No statistically significant difference between the control condition and the conditions in which rhDNase was added was observed (n = 3).

Table 3
Average log reductions (±SD) after tobramycin (4XMIC) treatment of biofilms pretreated with PBS or NaOCl.

Pretreatment	*B. cenocepacia* LMG 16656	*B. multivorans* LMG 13010	*B. cepacia* LMG 1222	*B. anthina* LMG 20980	*B. vietnamiensis* LMG 10929	*B. metallica* LMG 24068
PBS	3.92 (±0.19)	3.09 (±0.19)	4.01 (±0.13)	3.21 (±0.18)	4.24 (±0.17)	3.83 (±0.22)
NaOCl (60 μM)	4.67 * (±0.24)	4.25 * (±0.16)	4.86 * (±0.13)	3.38 (±0.30)	4.41 (±0.17)	5.25 * (±0.12)

* : statistically significant difference compared to PBS pretreatment ($p < 0.05$) (n = 3).

cough clearance and mucociliary transport [18]. In addition, rhDNase may have an impact on biofilm lung infections. By degrading eDNA present in the biofilm matrix it could sensitize bacteria to antibiotic therapy. In the present study we demonstrated the presence of eDNA in all Bcc biofilms investigated. The amount of eDNA differed among Bcc strains with the highest amount of eDNA measured for *B. multivorans* LMG 13010. These high levels of eDNA could possibly contribute to the virulence of the bacteria as eDNA has previously been shown to induce cationic antimicrobial resistance in *P. aeruginosa* and *S. enterica* [13]. rhDNase added at the start of biofilm formation significantly reduced the amount of eDNA in some Bcc strains. For two Bcc strains, *B. metallica* LMG 24068 and *B. vietnamiensis* LMG 10929 no decrease in eDNA concentration was measured after rhDNase treatment. Increasing the enzyme concentration could potentially increase the effect of rhDNase but we did not explore this further, as the concentrations used (50 μg/mL) were already at least 5 times higher than the concentrations measured in the sputum of CF patients after inhalation of 2.5 mg rhDNase I [19]. When added alone, rhDNase had no effect on the viability or dispersion of Bcc biofilm cells, which is in contrast with the observations of Tetz and Tetz for *Escherichia coli* and *S. aureus* biofilms [20]. The ability of DNase to disperse established *P. aeruginosa* biofilms has also been described [8]. However, although DNase I was capable of dispersing young biofilms, older biofilms were much less affected by DNase I, suggesting that other matrix components became increasingly important in stabilizing *P. aeruginosa* biofilms during growth. We subsequently investigated whether rhDNase could increase tobramycin susceptibility of Bcc biofilm cells. No increased bactericidal effect of tobramycin was observed if Bcc biofilms were formed in the presence of 50 μg/mL of rhDNase, nor if they were formed and treated in the presence of rhDNase, which is in agreement with tests previously performed in CF sputum [21]. Possible explanations are that, although eDNA is depolymerized by rhDNase, tobramycin can still bind to small eDNA molecules or that eDNA is not sufficiently degraded by rhDNase. Goodman et al. indicated that DNA binding (DNAB II) proteins contribute to the stabilization and structural integrity of eDNA within the matrix of different bacterial biofilms, including Bcc biofilms, which could protect eDNA from degradation by rhDNase [22,23]. Recently, it has been observed that antiserum directed against DNAB II proteins was capable of reducing *B. cenocepacia* biofilm mass, sensitizing the biofilm towards antimicrobials. In contrast, a rhDNase treatment increased the biofilm biomass [23]. A possible explanation for this is that smaller eDNA fragments, present in the biofilm matrix after rhDNase treatment, are increasingly stabilizing the Bcc biofilm matrix by cross-linking with other exopolymeric substances, including polysaccharides.

Cepacian is considered the major exopolysaccharide produced by the isolates of the Bcc [24]. A protective effect of *B. cenocepacia* EPS, and more specifically cepacian, towards ROS has been demonstrated [4]. By scavenging NaClO, the backbone of cepacian was degraded and acetyl substituents were cleaved off [5]. The loss of these acetyl substituents hampered the ability of cepacian chains to aggregate, reducing the viscosity of the polysaccharide network around Bcc cells [25]. To date, apart from PNAG, the production of seven different EPS by Bcc species has been demonstrated. The exact contribution of EPS in biofilm formation and protection against stress has yet to be determined [26]. Benincasa et al. already demonstrated a considerable decrease of the antibacterial activity of antimicrobial peptides in the presence of polysaccharides produced by the members of the Bcc [27]. To study the effect of cepacian on tobramycin bioactivity, we pretreated Bcc biofilms with 60 μM NaClO solution, in order to degrade cepacian, before adding tobramycin. When combined with tobramycin, we observed a significantly improved bactericidal activity of the latter against *B. cenocepacia* LMG 16656, *B. multivorans* LMG 13010, *B. cepacia* LMG 1222 and *B. metallica* LMG 24068 biofilms. On average, 30% less biofilm cells survived after combination therapy compared to tobramycin alone. The reactivity of ROS is most likely not limited to the degradation of cepacian but could also affect the integrity of other EPS. This is corroborated by our results which demonstrate a significantly increased bactericidal effect of tobramycin after NaClO pretreatment for *B. cenocepacia* LMG 16656, although this strain had previously been shown not to produce cepacian [28]. The use of a cepacian specific degrading enzyme, like the cepacian lyase isolated by Cescutti et al., would allow for better differentiation between the role of cepacian and other EPS in reducing the activity of tobramycin [29].

Table 4
Average log reductions (±SD) after tobramycin (4XMIC) treatment alone (controls) or in combination with dispersin B of 24 h-old biofilms.

Treatment	*B. cenocepacia* LMG 16656	*B. multivorans* LMG 13010	*B. cepacia* LMG 1222	*B. anthina* LMG 20980	*B. vietnamiensis* LMG 10929	*B. metallica* LMG 24068
Tobramycin	0.21 (±0.18)	0.71 (±0.12)	2.73 (±0.20)	2.11 (±0.26)	1.22 (±0.20)	2.57 (±0.15)
Tobramycin + dispersin B	0.46 * (±0.16)	0.72 (±0.16)	3.03 * (±0.27)	2.06 (±0.23)	1.38 (±0.18)	2.91 * (±0.14)

* : statistically significant difference compared to controls ($p < 0.05$) (n = 3).

The production of PNAG has been described for *B. multivorans*, *B. vietnamiensis*, *Burkholderia ambifaria*, *B. cepacia* and *B. cenocepacia* where it seemed to be important for biofilm formation and maintenance [7]. In addition, a mutant incapable of producing PNAG showed increased sensitivity to tobramycin, which suggests a role for PNAG in Bcc antibiotic resistance [7]. In contrast to these findings of Yakandawala et al., we did not observe a biofilm dispersing effect of dispersin B on Bcc biofilms. The relatively small biofilm killing effect of tobramycin monotherapy in this experiment is likely attributed to the buffer recommended for optimal dispersin B activity (100 mM phosphate buffer supplemented with 200 mM NaCl). As it has previously been noticed that tobramycin activity can be significantly reduced in high ionic strength solutions [30], this could explain the decreased bactericidal effect of tobramycin. Nevertheless, the combination of dispersin B (100 μg/mL) with tobramycin significantly increased the latter's bactericidal effect against *B. cenocepacia* LMG 16656, *B. cepacia* LMG 1222 and *B. metallica* LMG 24068. The combination therapy resulted in a 10–45% decrease in biofilm cell numbers compared to tobramycin treatment alone for these isolates.

5. Conclusion

We investigated the *in vitro* bactericidal activity of tobramycin in combination with matrix degrading substances. rhDNase did not show any additional effect on antibiotic killing which could be due to a biofilm stabilizing effect of small eDNA fragments present in the biofilm matrix after rhDNase treatment. EPS degradation, by NaClO pretreatment, significantly increased the bactericidal effect of tobramycin towards some of the Bcc biofilms tested, as did selective PNAG degradation by dispersin B.

Acknowledgments

This research has been funded by the Interuniversity Attraction Poles Programme initiated by the Belgian Science Policy Office and by a concerted action grant by Ghent University.

We thank Kane Biotech for providing dispersin B and Petra Rigole and Laure Pape for their excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2013.07.004.

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